#14 6/10/02

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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Cole et al.		
Serial No.	:	09/508,710	Examiner:	David H. Kruse
Filed	:	July 10, 2000	Group Art Unit:	1638
For	:	NEW PLANT GENES		

DECLARATION UNDER 37 C.F.R. § 1.131

Ian Cummins declares as follows:

1. I am an inventor of the above-identified patent application.

2. Prior to August 11, 1997, the invention described and claimed in the subject application was completed as evidenced by the following.

3. Prior to August 11, 1997, Ian Cummins isolated RNA encoding the glutathione transferase subunits of the present invention. A copy of the relevant lab notebook pages in which Ian Cummins described this work are attached hereto as Exhibit A. The dates shown on these pages are redacted.

4. Prior to August 11, 1997, Ian Cummins synthesized cDNA library from the isolated RNA described in Exhibit A and purified the cDNA library. A copy of the relevant notebook pages in which Ian Cummins described this work are attached hereto as Exhibit B. The dates of Exhibit B are redacted.

5. Prior to August 11, 1997, Ian Cummins sub-cloned the cDNA library into expression vectors and transformed *E. coli* cells with the expression vectors. A copy of the

relevant notebook pages in which Ian Cummins described this work are attached hereto as Exhibit C. The dates of Exhibit C are redacted.

6. Prior to August 11, 1997, Ian Cummins checked the cDNA library expression vectors for the presence of glutathione transferase subunits by binding to a glutathione resin and by western blot analysis. A copy of the relevant notebook pages in which Ian Cummins described this work are attached hereto as Exhibit D. The dates of Exhibit D are redacted.

7. Prior to August 11, 1997, Ian Cummins showed by western blot analysis that certain cDNA's expressed glutathione transferase subunits. A copy of the relevant notebook pages in which Ian Cummins described this work are attached hereto as Exhibit E. The dates of Exhibit E are redacted.

8. Prior to August 11, 1997, Ian Cummins sequenced the cDNA encoding for glutathione transferase subunits and showed that they were novel DNA sequences encoding novel glutathione transferase subunits. A copy of the relevant notebook pages in which Ian Cummins described this work are attached hereto as Exhibit F. The dates of Exhibit F are redacted.

Ian Cummins

Date: May 30th 2002

IAN CUNNS.

UNIVERSAL OFFICE SUPPLIES

DUPLICATE BOOK

IAN CUNNS.
WHOLE CST
BOOK 3.

BIOLOGICAL SCIENCES,
UNIVERSITY OF DURHAM,
DURHAM,
DH1 3LE,
UK

Re-Order Code DUPA4U

Concentrate gel isolated protein + run an aliquot on a gel.
→ ≈ 150 μl. Run 5 μl on gel.

Freeze - dried over-expressed from 10 + 11 (originally 2 ml of each 2 × 1 ml tube)

1 tube of each → -20°C for next time.
1 tube of each → 200 μl H₂O. So use 2 μl + 1 μl on gel.

M10 mix with 25 KD isolated protein.

Use 20 μl of 18H with 2 μl of resuspended protein.

Gel.
1) SDS 7B.
2) Gel isolated protein 5 μl.
3) Marker.
4) 18H 20 μl.
5) 18H 20 μl.
6) 24P 20 μl.
7) 2 μl #11 Overexpressed + 20 μl 18H
8) 1 μl overexpressed. #11
9) 2 μl overexpressed #11
10) 18H 20 μl.

Run @ 150V + silver stain

Blot OK. 24 KD is present.
Overexpressed band is 26 KD.

Prepare reagents / glassware / plasticware for RNA prep. from stored stocks.

<u>TRIZOL PREP.</u>
CHLOROFORM ONLY
IPA
75% EtOH

10 vols. trizol.
-20

1) Homogenise 10-20 vols. 5 g (50 ml)
2) 5 min @ R.T.
3) Add 0·2 ml $CHCl_3$ / ml TRIZOL (10 ml)
4) Shake 15 sec. RT 2-3 min.

RNA protocol cont.

RNA was extracted with P/C/IAA pH 4 x 1
C/IAA x 1
+ EtOH ppt'd,
→ 1ml, Scan 1/100
A 260 = 0·36
A 280 = 0·23
= 1·56

8) Inc. RT 10 min.
9) 12,000g 10 min. 4°C. → RNA pellet.
10) Wash 75% EtOH (1ml 2 wash for 1 ml mix)
11) Vortex + cent. 7,500g 5 min 4°C.
12) Air dry resuspend in DEPC H₂O + check it out.

A1 = 40μg/ml. 1·46μg/ml
RNA → -80°C. Aliquots.

Poly(A)$^+$ selection.

Buffer to make – 1x Washing Buffer. 10mM TRIS-HCl pH 7·5
150mM LiCl
1mM EDTA.

75μg RNA → ≈ 1·5μg A$^+$.

1) Start with 75μg RNA in 100μl.
2) → 65°C 2 min.
3) Use 200μl Beads + add to Epp. in Magnet. After 30 secs remove SN + wash with 100μl 2x Binding Buffer. (PINK).
4) Then remove from magnet + add 100μl 2x Binding Buffer
5) Add RNA + mix – stand 5 min RT.
6) Magnet 30 secs. Remove SN.
7) Wash 2x 200μl wash buffer.
8) Add elution buffer → 65°C, 2 min → magnet + remove SN to new tube.
Can add RNasin + store -80°C.

Wavelength: 280.0

Scan directory: VI
Start wl: 220.0 nm
End wl: 300.0 nm
Overlay scans: [No

Function	Reading
Scan	0.22848

] Method name: A:\DEFAULT
] Autosave name: [A:\]SCANS
e: 1 Sampling device: None
[sec] Read average time: 0.50 sec

A:\WORK_003

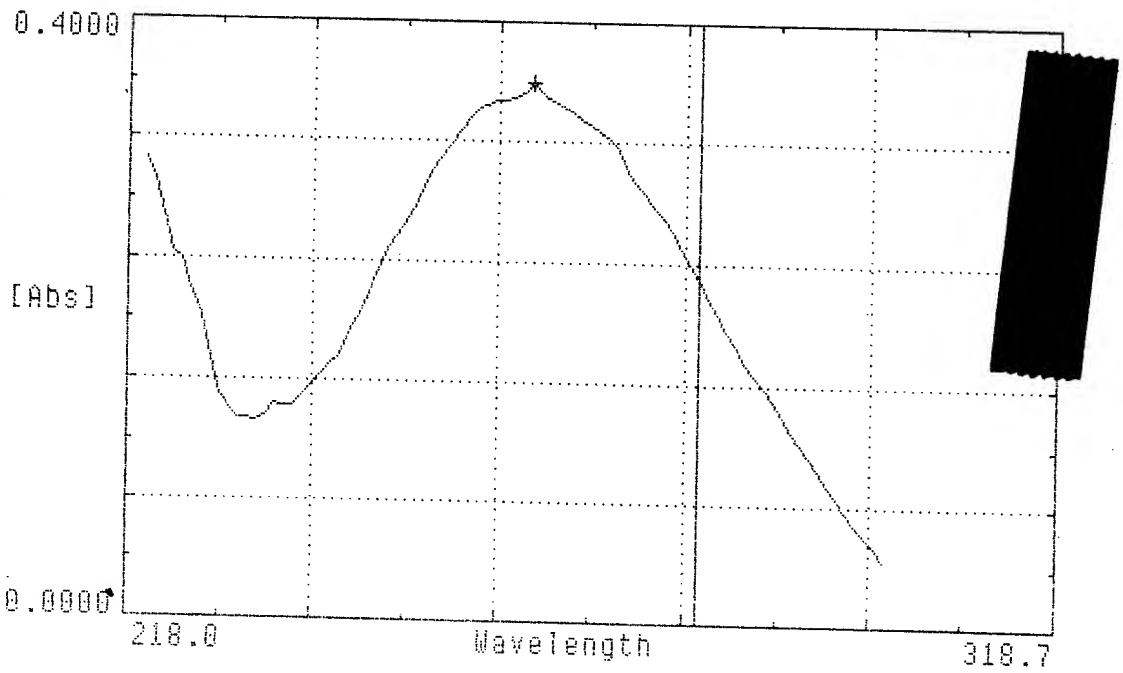
Print ↓ ↑ ← → Exit

A:\WORK_003 Scan

wl	Abs	Pick
262.0	0.3603	pk

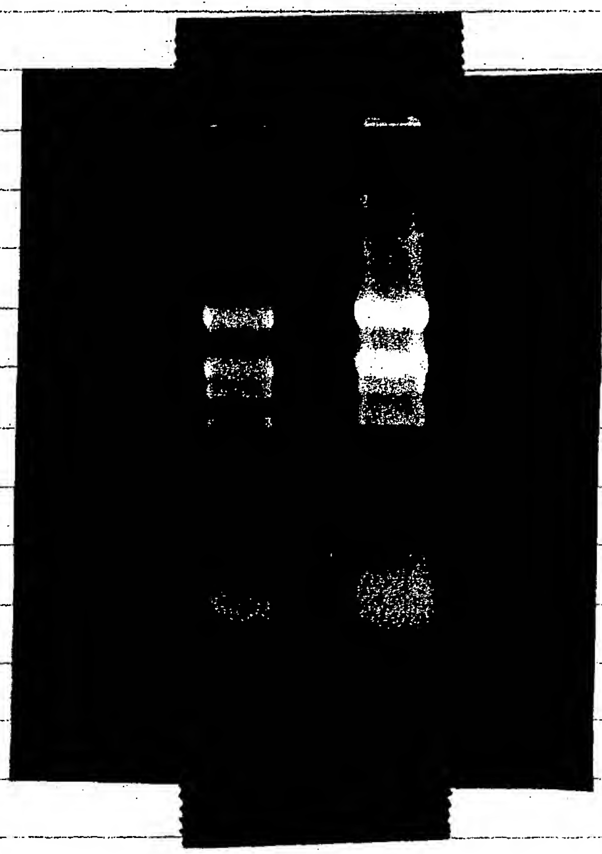
Zoom ZoomOut Trace Function Autoscale Annotate Print

Functions: Scan



Run 2µl + 5µl RNA + eq. vol of 100% formd on 1.2% agarose TBE gel, 75V 1hr.

Stain 15min
EtBr 0.5µg/ml.



RNA @ 1.44 µg/µl.
for 75µg

52µl + 48µl H2O.
→ 100µl per column.

tiny any 100µl read
= 144µg.

→ ≈ 1→2µg.
∴ Do 5 cols
for ≈ 5µg.

Looks OK – some poly(A)+.

Prepare wash solution (1M Tris 7.5 stock, DEPC treat EDTA).

		Stock	For 10ml	1ml
1x WASH Buffer –	10mM Tris-HCl pH 7.5	1M	100µl	.10
	150mM LiCl	8M	188µl	18.8
	1mM EDTA	0.5M	20µl	2
				969

308µl
9,692µl
10,000µl.

Pooled 100µl RNA x2
130µl RNA (#3) eluted = 8µl each. (→24µl)
3×8µl aliquots.
→ -80°C.

Also pooled unbound → -80°C.

[redacted]

Wavelength Scan
ReadSamples   Tabulate   +-*Scans   Scatt

Scan directory: VIEW   Autoprint: [No
Start wl: 220.0 nm   Autosave: [No
End wl: 320.0 nm   Scans per samp
Overlay scans: [No]   Interval: 5.00

A:\WORK_001   5μl of 40

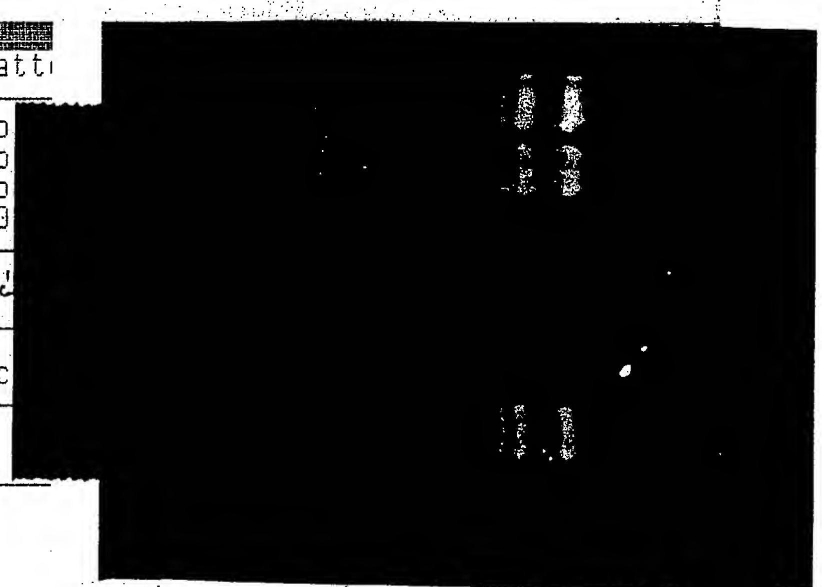
Print ↓ ↑ ← → Exit   Zoom   ZoomOut   Trac

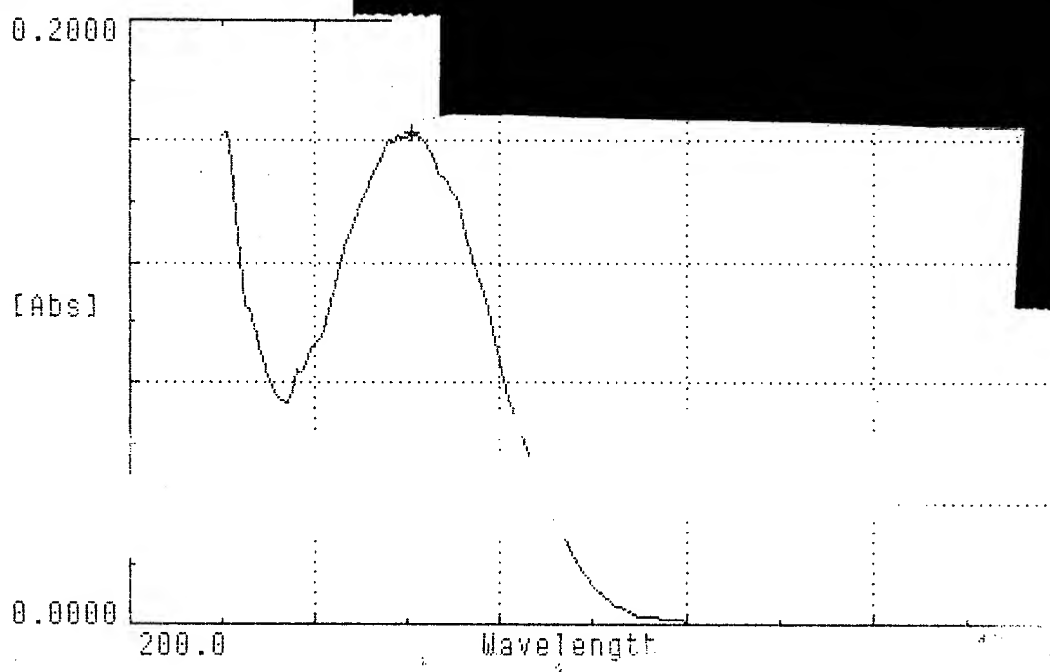
A:\WORK_001   Scan   Functions: Scan

wl	Abs	Pick
261.0	0.1627	pk
280	0.0865	

1.88

A = 3.254
= 130μg/ml
= 130ng/μl
10μl = 1.3μg
= 4.5μg in 35μl





So process the rest of the RNA as a week tomorrow. But run 5μl on a gel with 5'

[redacted]

First injections for rabbits 26 + 27
↓ ↓
Pierce (26) Gt 27 Hom

Use all of sol. made HA for 26, + 2 tubes of our own (#10 + 11) for #27. → 1½ ml + 1½ ml FCA.

Prepare more poly(A)$^+$ from total RNA.

for 1 ml.

Make 2 x Binding Buffer. 20 mM TRIS-HCl pH 7.5 (1M) 20 ml
1 M LiCl (8M) 125 ml
2 mM EDTA (0.5M) 4 ml
H_2O 851 ml

for 2 ml 2 x Binding Buffer 40 ml TRIS (1M)
250 ml 8M LiCl.
8 ml 500 mM EDTA
1702 ml H_2O (851 x 2)
2 ml

2 x 200 ml aliquots of total RNA processed → 10 µl each

Pool all the A$^+$, scan + run on gel.
Also did Jil prep on 200 ml tot. RNA → 10 ml, Run 1 lane.

Gel. 1) Dye.
2) –
3) Tot. 2 ml
4) 1st Pool A$^+$ 2 ml, → 80V. Stain.
5) 2nd prep. 1 ml,
6) –
7) 1st Bot. unbound 5 ml.
8) 2nd " " 5 ml.

cDNA synthesis from ≈ 5μg poly(A)$^+$ RNA.

Followed the stratagene protocol & then to precipitate for 1½ day @ -20°C (at lunch tomorrow)

12. 6. 96.

At [illegible] 96.

13. 6. 96.

Pelleted cDNA 60 min, 4°C, 10,000g.
washed pellet 70% EtOH, dried & resuspended in 8μl EcoR1 adaptors. Ligated O/N as per protocol 8°C.

14. 6. 96.

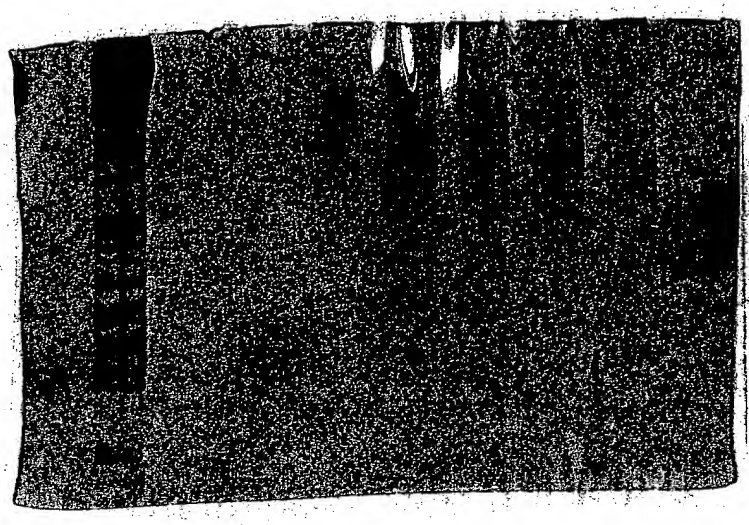
Kinase & Xho1 digestion of cDNA prior to spin column.

Made 6% acrylamide gel in 1 x TBE for fraction analysis.

Stock acrylamide is 40%

for 10 ml	1 ml	10 x TBE
	1.5 ml	Acrylamide / Bis.
	7.5 ml	H_2O
	10 ml.	

100μl APS 10%, 10μl TEMED.



Eluted cDNA was pooled then Sephacryl S-500 column.
6 fractions taken = 60 µl.

5 µl of each → gel.

Gel run in 1× TBE, 150 V ≃ 45 min till BPB at bottom

Stained in BioRad after stain.

Gel.			
1)	—		
2)	λAt 5 µl.		
3)	—		
4)	Frac 1	very GHR.	
5)	2	800 bp → 3 kb	use 2 + 3.
6)	3	500 bp → 3 kb	
7)	4	"	
8)	5	200 bp → 3 kb.	
9)	6		
10)	—		

fractions extracted with P/C ×1.
C/IAA ×1 + EtOH ppt'd. −20°C
over the weekend.

Pellet the precipitating DNA 10,000 g, 4°C, 1 hr.
Resuspend the DNA pellet in 4 ml st. H_2O.
Quantify on Ethidium Bromide Plate.

Set up ligation O/N 12°C.

Prepare host strain for packaging reaction.

XL1B MRF' - plate out onto LB Tet Agar. (12.5 mg/ml)

Inoculate 50 ml LB Tet 10mM $MgSO_4$, 0.2% maltose.
↳ 37°C 4-6 hr OD 1.1.
↳ Pellet 500 g 10 min.
↳ 25 ml 10mM $MgSO_4$.

VCS 257 → LB
SOLR → LB kan (50 mg/ml)

Make 20% maltose in st. H_2O + 2 filter sterilize.
10mM $MgSO_4$ - autoclave.
st. H_2O.
Kanamycin stock (50 mg/ml).

20% Maltose – for 50ml 10g + Zero Stalin.
make 25ml – 5g.

		for 50ml LB Tet Mgml
LB let plates – 500ml –	5g NaCl	500mg NaCl
	5g Tryptone	500mg Trp.
	2.5g Yeast Extract	250mg YE
	10g Agar.	125mg $MgSO_4.7H_2O$
	↳ pH 7.0	PH 7.0

$MgSO_4 = 246.5$. 10mm = 2.5g/l

		250ml	
LB top – 500ml	5g NaCl	2.5	
	5g Tryptone	2.5	10mM $MgSO_4$
	2.5g Yeast Extract	1.25	1.25g → 500ml
	3.5g Agarose	1.75	
	↳ pH 7.0		

NZY Agar plates – 500ml –	2.5g NaCl	
	1g $MgSO_4.7H_2O$	
	2.5g Yeast Extract	
	5g Casein Hydrolysate	
	7.5g Agar.	

NZY top – 500ml –	2.5g NaCl	
	1g $MgSO_4.7H_2O$	
	2.5g Yeast Extract	
	5g Casein Hydrolysate	

Panel 3 dilution of NZTA 51 TRL [redacted]
20 LB ... plates

SM buffer - 200ml.

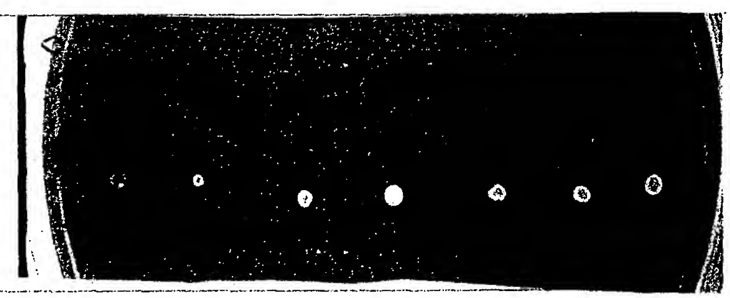
1.16g NaCl.
400mg $MgSO_4.7H_2O$
10ml 1M TRIS (pH7.5) 1.21g TRIS
1 ml 2% Gelatin 20mg Gelatin.

Fractions 1,2 & 3 were pooled into 5μl H_2O, since these all had no small cDNA.

0.5μl was spotted onto EtBr plate with DNA standards

Appears to be plenty of cDNA. Call it 150ng/μl.

Set up ligation & store the rest of the cDNA. -80°C

[redacted]  [redacted]

~100ng insert + 1μg vector.

Ligation 1
0.75μl cDNA
0.5μl 10 x Buffer
0.5μl rATP
1μl Uni-ZAP
1.75μl H_2O
0.5μl ligase.

Ligation 2 Dilute cDNA x3 use 1μl.
1μl cDNA.
0.5μl 10x
0.5μl ATP
1μl ZAP
1.5μl H_2O
0.5μl ligase.

Grow plating cells - 50 ml LB Mg + 0.2% maltose + 12 μg/ml Tet.
→ add colony of XL1B MRF', → 37°C 200 rpm.

Lysates → 4°C.

Today just make plating cells + do packaging etc. tomorrow because of exact tubes.

Cells grown to OD 0.598.
Pellet 1500g in 2 × 25 ml aliquots @ 4°C
Resuspend in 15 ml 10mM $MgSO_4$ → OD [illegible]
Store on ice @ 4°C.

Dilute tomorrow to package.

Packaging of λ bring. 3 μl of lysate 1 + 3 μl of lysate 2 were packaged with 1 tube each of Gigapack III for 90 min.
Remainder of lysate → -80°C.

Make 1 ml 0.5M IPTG in water.
1 ml X-Gal 250 mg/ml in DMF.

IPTG = 238.3   1M = 238.3 mg/ml
0.5M = 119 mg/ml

Plating cells OD = 1.4 v = 15 ml.   i.e. → 42 ml for OD 0.5
1 ml → 2.8 ml
0.33 ml → 0.933 ml
0.33 ml + 0.6 ml.

Plated packaged 1 + 2 libraries on NZY X-GAL IPTG as in Stratagene manual, in 2.5ml NZY TOP (3ml better)

1μl Neat, 1/10, 1/100 + 1/1,000 for 1 + 2.

Dilutions made in SM. Stored at 4°C.

Plates → 37°C O/N.

Also transformed BL21-DE3 with plasmid from pET/GST 27 #1 - plasmid prep from 9/4/96. Use this to make new protein prep.

Undiluted plating cells → on ice @ 4°C room.

Tomorrow - amplify library + make plate for screen also.

GST pET transformations OK → 4°C for next week

Library titres OK. Still % of blues on neat plates.

Lib #1 1/10 = 73 plaques 0 blues 730/μl for 500μl = (365,000 pfu) or 1,150,000

2 1/10 = 178 " 3 blues 890,000

1,050,000

Also have other lysate to be packaged.

≃ 2,700,000 pfu.

≃ 1 ml of library.

for immunostain — grow 37°C 3-4 h.

N. meningitidis in Dam 10% — dry on paper.

Overg. ≥ 4 h 37°C.

Remove lid 20 min.

Cool, mark & save.

western blot.

Peak → 4°C.

Plate library on 2 dishes for amplification.

Re-count of 1/100 plates for library titre.
Blue background in small = 2%

1 1/100 = 33, 0 blues 3,300 pfu/pl 1,650,000 pfu
2 1/100 = 22, 2 blues 2,200 pfu/pl 1,100,000 pfu
i.e. 2,750,000 pfu in 1ml

Use 3ml top agar for 90mm plate 64cm²
Bioassay dish = 591cm² ≡ 27.7 ml top agarose
use 30ml, i.e. 10x vols. of plate

30ml top agarose.
2ml plating cells @ OD 0.5.

By observation - 2 ul library / 90 mm plate is OK ≡ 20 ul for dish
20 ul = 66,000 pfu 1 on plate more densely i.e. 170,000
= 44,000 pfu 2

plating cells - 330 ul + 660 ul MgSO4
for 4ml 1.32ml 2.64 ul

= 51 ul lib 1
= 77 ul lib 2

#1	#2
2ml cells	2ml cells
51 ul 1/2 lib 1	77 ul 1/2 lib 2
30ml TOP Agarose	30ml TOP Agarose

Grow o/v 37°C, overlay with 30ml SM, rock o/N 4°C.
Remove SM, wash plate 7ml SM pool. $CHCl_3$ to 5% v/v, mix RT 15min
Pellet 10 min 500g. SN → $CHCl_3$ 0.3% v/v 4°C.
Store -80°C 7% DMSO.

Make 2 x 200 ml LB for overnight ferm.

for 400 ml LB 4g NaCl
4g Tryp
2g Yeast Ext → pH 7.0

Make Ampicillin @ 100 mg/ml.
Carbenicillin @ 100 mg/ml.

Set up 2 x 5 ml o/n cultures from plate. 100 µg/ml carbenicillin

Wash the ampicillin colony plates in 30 ml SM with shaking @ 4°C for 6 h. Then add $CHCl_3$ to 5%. → RT 15 min.

Pellet 10 min, 500g.
SN → Falcon tube + 0.3% $CHCl_3$.

Also innoculate 2 x 200 ml LB carb 100 1 for 200 ml o/n.
1 for colony.

Grow 2-3 h x o/n 180 rpm to 1mM.

Lib 1 → 32 ml. (+1.6 ml $CHCl_3$) → 29 ml 87 µl $CHCl_3$
Lib 2 → 29 ml. (+1.45 ml $CHCl_3$) → 27 ml 81 µl $CHCl_3$

PCR on 5 plaques from plate 1, 5 from plate 2.

T3 + T7 primers are @ 15 pm/µl.

Pick phage → 100 µl SM – where use 5 µl for template.

PCR mix

9.0 µl	25 mM $MgCl_2$
5 µl	10x Buffer
8 µl	1.25 mM dNTPs
3 µl	T3
3 µl	T7
5 µl	Template
0.5 µl	TAQ

– use MS mix. '11x'

		½ rxn
4.5 µl	MS mix	2.25
5 µl	Temp.	2.5
0.5 µl	TAQ	0.25
3 µl	T3	1.5
3 µl	T7	1.5
34 µl	H_2O	17
50 µl.		25 µl

Result – run on gel – all have 800bp band + additional faint bands 500bp → 2kb.
PCR components contaminated?
Do another PCR maybe with different primers to check.

Master mix for 11 tubes =

24.75 µl	MS 11x
2.75 µl	Taq.
16.5 µl	T3
16.5 µl	T7
187 µl	H_2O
247.5 µl.	

22.5 µl Mix + 2.5 µl Temp.

PCR – 25 x 94 – 1, 50 – 1, 72 – 2

Plating cells grown o/N in 50 ml LB mg. Tet. 30°C.

Pelleted → 10 ml 10mm $MgSO_4$ → OD 2.1.

Take 1ml + 3 ml 10mm $MgSO_4$ → OD 0.5 for plating for immunoscreen.

(OD 0.5)
Screen 1 big dish — 2 ml plating cells }
40 ml Lb2 } → [redacted] .n.
25 ml Lb1 }
30 ml TOP Agarose.

on e 10-15 am.
Grow 3-4 h, overlay with filter in 10mm IPTG.
Grow 3-4 h & remove filter.

IPTG = 238.
10mm = 2.38 mg/ml.
for 100 ml — 238 mg, in st. H_2O.

After 3½ h plaques just visible. Overlay with N/C + IPTG for 4 h. Lift & (store in TBS o/N.) Detect tomorrow.

D.D. ran some test protein from Sally's aequorin. Looks OK.

Filter was marked in each corner. Lifted & rinse in 1×TBS → Block it up in 200 ml 3% Marvel, 1×TBS.

Inc o/N 4°C 21F $\frac{1}{5000}$ for 200 ml = 40 μl Ab.

[redacted]

26

[redacted]

Wash filter as usual. → 2'y Ab 1/5,000 1h.
Wash as usual.
Wash 100 mM TRIS 9.5 + develop.
→ 15' +ves.

Run gel & blot for new Abs.

1) SDS FB.
2) 24P 20 ℓ
3) 14H 20 ℓ. Run c 150V
4) 23 H 20 ℓ. Blot.
5) Overexpressed 5 ℓ. Store blot -20°C until Abs ready.
6) SDS FB.
7) 24P Results — 27 - currently picks out band ≈ 3 wt.G.m.
8) 14H No reaction with overexpressed protein
9) 23H 26 - nothing.
10) Overexpressed. ∴ Boost & check again

Abs. arrived, so block c inc c Ab. 1/1,000 O/N.
Recovered ≈ 8 ml of each. → -20°C 1ml aliquots.

Picked 17 1'y plugs → 1ml SM + 20 ℓ $CHCl_3$.
Vortex & store 4°C. Assume titre ≈ 6 x 10^7 pfu/ml.
i.e. 6 x 10^4 pfu/µl.

Develop western blots. Wash as usual. 2'y Ab $\frac{1}{10,000}$, 1hr.

Secondary screen — assume 1'y stocks are c 6×10^4 pfu/μl

i.e. 10 μl = 6×10^5 pfu.

Need 6×10^1 – 6×10^2 pfu for 1 plate.

i.e. 10 μl of 10^{-3} – 10^{-4} stocks to OK for 2'y screen. 10^{-1} is 1 μl per ml.
+ dilute x 10 for 10^{-4}.

200 μl cells, 10 μl phage, 3 ml TOP Agarose.

↳ 37°C 3½ – 4 hr.

Overlay with N/C + IPTG + inc. 37°C o/N.

10^{-4} plates OK. Lift N/C, wash TBS, block 3% M.P/TBS inc. 2IF $\frac{1}{5000}$ 1 hr. for 400 ml = 80 μl.

		-3	-4		-3	-4
Pick-	1	✓	–	9		✓
	2	✓	–	10		✓
	3	✓	–	11		✓
Pick 10^{-3} plates	4		✓	12	✓	–
for 10^{-4} – very	5		✓	13	✓	✓
	6	✓	–	14	✓	–
	7	–	–	15	✓	–
	8		✓	16	✓	–

Pick 2'y screen.

where possible pick singles for rescreen & cryoing.

If too dense, then pick all patches from 1 plate, pool & re-screen next week.

Also Hek-GH culture from frozen old pellets & compare CST II homologue.

Use pellets by counter ~ 10 ml tube. Pellet debris & do CDMB assay.

Clear = 0.051

SN > 0.105.

Apply to Hek-GH as well. Assay all fxns for CDMB activity.

Did not bind! So for the moment skin this & use frozen stuff for Ab.

Procedure for Single-clone excision.

1) Streak XL1B MRF' on LB tet. (12 µg/ml)
SOLR LB kan (50 µg/ml)

2) Grow o/N colony of XL1BMRF' tet Mal MgSO4.
SOLR + LB kan.

Make 100 ml LB + 500 ml flask.

Need. Ampicillin Plates

5 ml LBs

o/N liquid culture of XL1BMRF' maltose/Mg/Tet
+ SOLR kan

Pellet 500 g 15 min + suspend in 10 mM MgSO4
to OD of 1.0.

Ex-assist is @ 10^7 pfu/ml. — use 1 µl.
Assist phage pfu are @ 10^4 pfu/ml. — use 100 µl + 150 µl SM.
200 µl XL1BMRF' @ OD 1

↳ 37°C, 15 min + filter free

Add 3 ml LB + inc. 2½ h 37°C with shaking.

↳ 70°C, 20 min — 1000 g 15 min. Keep SN.

for OD1

SOLR (25ml MgSO4) OD = 2.02 → 50ml.
XL1B (25ml MgSO4) OD = 2.5. → 62.5ml.

Perform plaque assay on the 12 single plaque picks isolated last Friday. As per protocol, took 10 μl & 1 μl of excised phagemids with 200 μl SOLR cells & plated different amounts of this onto LB Amp plates. → 37°C o/N.

Plenty of colonies on all the plates. Grow these up o/N in LB AMP KAN. for plasmid preps tomorrow.

Today – re-screen the 1° picks I could not purify by 2° screen, i.e # 7, 3, 12.

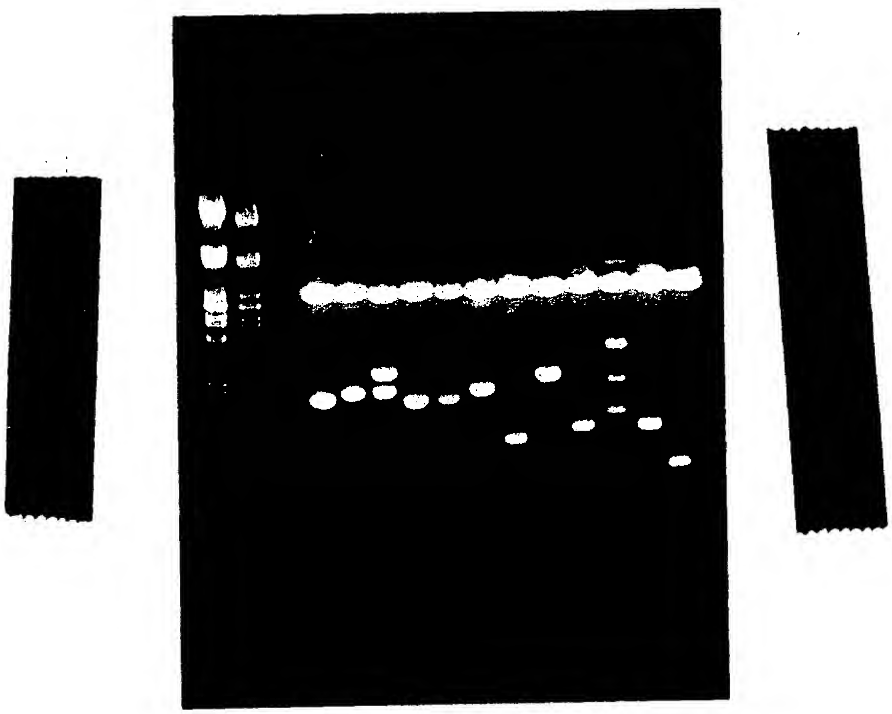
Dilute to 10^{-3} & 10^{-4} & use 10 μl phage with 100 μl cells OD 1 & 3 ml top agarose.

Incubate 4 hrs, overlay with N/C disc + IPTG & inc O/N.

Also – gel purify 24 kD band on 2 0.75mm 15% Tris/Tricine gels.

Load 100 μl of 9/2 # 10 on each. Fix 15 min. Stain 10 min – excise band & inc. in Porage buffer o/N.

↳ Bands not clearly resolved – must be overloaded. So no use for these gels.



New Miz. preps. from 12 O/N SOLR cultures.

3ml culture → 50µl DNA.

Digest 5µl + 30µl EcoR1 / Xho1.

5µl DNA		39µl 10×
3µl 10×	for 13 make mix	13µl EcoR1
1µl EcoR1		13µl Xho1
1µl Xho1		260µl H₂O
20µl H₂O		
30µl		+ me 25µl for each digest.

Run a 1% Agarose / TBE gel.

Digest λ/Pst1 marker. λ is 0.5µg/µl. for 50ng - 100µl.

100µl λ (50ng)
20µl 10× Buff.
10µl Pst1
70µl H₂O
200µl
↳ 37°C 1 L.

Clones are 1, 2, 4, 5, 6, 8
9, 10, 11, 13, 14, 16.

Rerun #3-12 O/N.
No +ve for #7.

Miz. DNA → −20°C for later seqing.

Check plasmids 1 & 2 prior to sequencing.

1 µl → 10 µl. & run 1, 2, 4 µl on gel.
2 & 6 µl λ Pst + 100 ng/µl.

200 ng λ Pst → 12.5 ng 3 kb.
600 ng λ Pst → 37 ng 3 kb.

Sequencing DNA 5 µl of 100 ng/µl per reaction.

1 µl $\frac{1}{10}$ ≃ 37 ng. i.e. 370 ng/µl of stock

Take 1.3 µl of stock → 5 µl. Do F & R on 1 & 2
& do further restriction mapping & expression studies.

1.3 µl DNA + 3.7 µl H_2O.

Rescue clone for plasmids 12 & 3 (A & B for both).

1 µl of excised phagemids + 200 µl SOLR cells → 37°C 15 min.
Title on dished plates (Amp 50/50) & grow O/N.

Make 200 ml LB Agar.		100 ml LB (20 × 5 ml)	
2 g	NaCl	1 g NaCl	1 µl SN
2 g	Typtone	1 g Typ	split
1 g	YE	0.5 g YE	
2 g	Agar.		

↪ pH 7.0 & autoclave.

Run # 9 8.2.96 on 2 x 15 %. SDS gels.

176 µl + 176 µl buffer split between the 2.

Fix 10 min, 45 % methanol, 10 % HAc
Stain B+B 30 min

Excise bands → 500 µl Ponceau buffer – homogenise →
37°C O/N.

Clones 1, 2, 4, 5, 6, 8, 9, 10, 11, 13, 14 + 16 induced.

Inoc. 5ml LB Amp/Kan with 100 µl of parent clone.
Grow to OD 0.6 → 1 + add IPTG to 1mM. Grow
3 h.

– In fact – grow for 2½ h, still OD ~ 0.2. So add IPTG
+ grow for an additional 6 h. Pellet + keep until Monday.

Excise probe → 40 µl. Add 20 µl dH2O → -80°C.

Prepare 2 gels of CST clones from prep. used for WI2
DNA preps. Also keep 1 µl of culture + pellet for
control c.f. induced cultures.

700 µl culture + 300 µl 50 % Glycerol.
350 + 150.

65

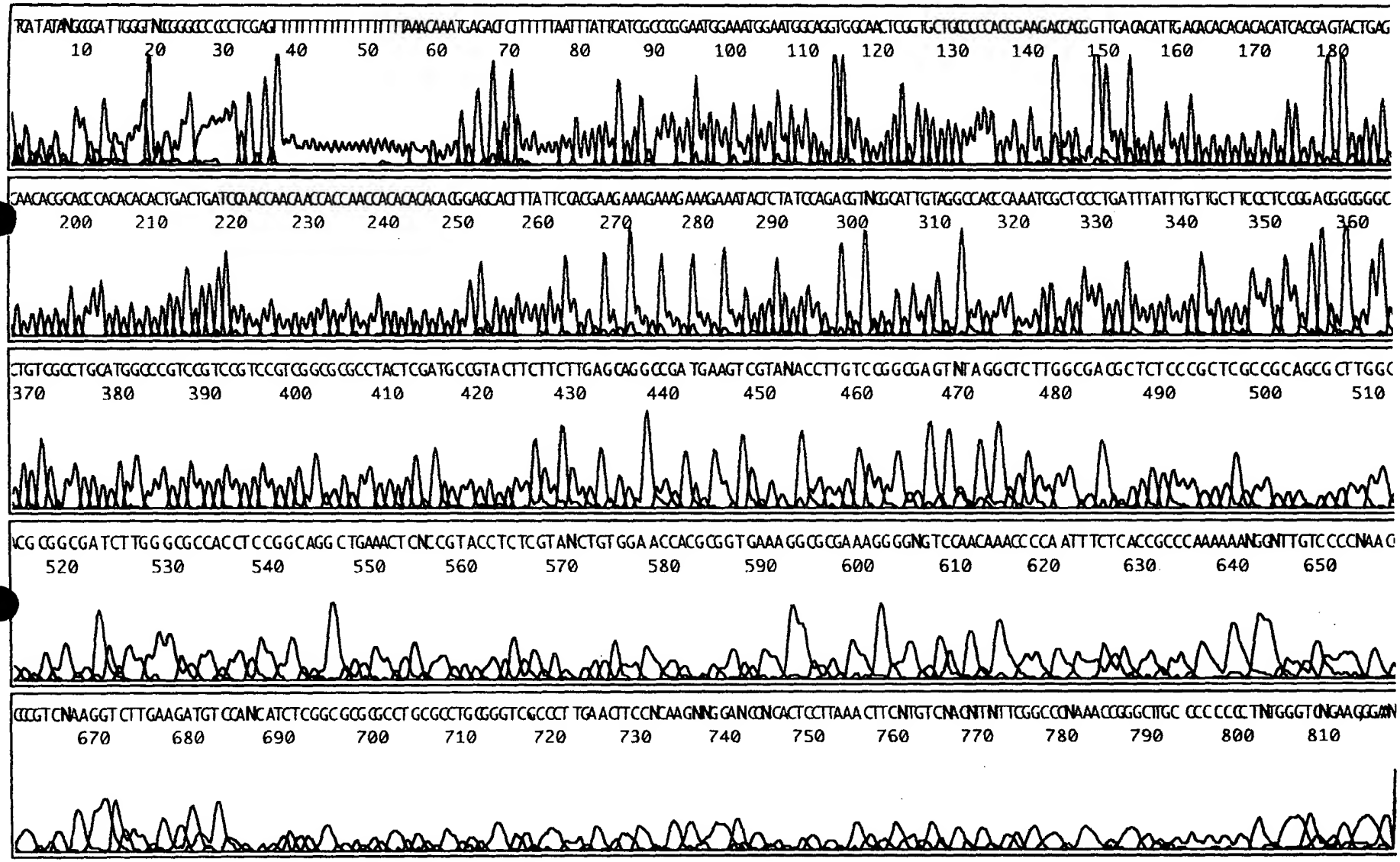
ABI PRISM

Model
Version 3.0
ABI50
Version 2.1.1

040796_05
Primer -21M13
IC: WT 25.2
Lane 5

Signal G:164 A:214 T:60 C:76
DT4%Ac{A Set-AnyPrimer}
Instrument 901356 JB.
Points 1160 to 8600 Base 1: 1160

Spacing: 9.10{



ABI PRISM

Model
Version 3.0
ABI50
Version 2.1.1

040796_09
Primer reverse
IC: GTT 25.2
Lane 9

25.2

Signal G:158 A:196 T:43 C:69
DT4%Ac{A Set-AnyPrimer}
Instrument 901356 JB.
Points 1160 to 8000 Base 1: 1160

Pag

Spacing: 9.2